REMARKS

I. Preliminary Remarks

Claims 31, 43, 67 and 73 are amended herein. Support for the phrase "improves or stabilizes one or more clinical features of a CNS disorder in a patient" can be found in the specification as originally filed, for example, at page 12, lines 28-31. Accordingly, no new matter has been added by the amendments of claims 31, 43, 67 and 73.

Applicants reserve the right to claim the subject matter of any original claim or any described invention in this application, or in related applications, such as continuing applications.

II. The rejections under 35 U.S.C. § 112, second paragraph, should be withdrawn.

The Examiner variously rejected claims 31, 33-46, 48 and 72-73 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. Applicants request reconsideration of the rejections in view of the amendments made herein and the following remarks.

The Examiner asserted that claims 31 and 73 are indefinite for reciting the phrase "a clinically recognized improvement or stabilization of one or more clinical features of a CNS disorder in a statistically significant number of patients." The rejection is moot because the phrase has been deleted from the rejected claims. Claims 31, 67 and 73 now recite "improves or stabilizes one or more clinical features of a CNS disorder in a patient." The specification as originally filed indicates that clinical features of a CNS disorder that can be monitored include, e.g., cognitive function, memory, behavior, language skills, motor skills or rigidity of the patient. See page 12, lines 28-31. Methods and criteria for determining whether one or more of these features improves or stabilizes in a patient was well known to one of skill in the art prior to the filing date of this present application. See, for example, The Merck Manual, 17th Edition (1999), pages 1344-1346 and 1390-1398, set forth in Appendix A.

The Examiner also asserted that claim 43 is indefinite for "reciting an Aβ polypeptide comprising amino acids 1-39 of SEQ ID NO: 1 while being dependent from claims 70 and 72, which specifically require substitutions with Aβ." The rejection of claim 43 based on its dependency on claim 70 is moot in view of the amendment to claim 43. The Examiner is incorrect that claim 72 requires substitutions within Aβ. Claim 72 makes no reference to such substitutions in Aβ. Accordingly, the rejection should be withdrawn.

The Examiner further asserted that claims 45 and 46 allegedly lack antecedent basis because these claims recite an Aβ polypeptide with substitutions wherein the base claim 31 is allegedly known in the art to be limited to an Aβ polypeptide defined by SEQ ID NO: 1. Applicants disagree with the Examiner's interpretation of the term "Aβ polypeptide" recited in claim 31. According to MPEP § 2111, the claims must be given their broadest reasonable interpretation *consistent with the specification*. The specification as originally filed defines an "Aβ polypeptide" as "(1) the naturally occurring human Aβ of SEQ ID NO: 1; (2) polypeptides having one or more substitutions or insertions in the amino acid sequence of the naturally occurring human Aβ polypeptide that retain the ability to cross the BBB [blood brain barrier]; and (3) fragments of (1) [naturally occurring human Aβ of SEQ ID NO: 1] that retain the ability to cross the BBB." See page 4, lines 9-14, of the specification. Accordingly, the broadest interpretation of the term "Aβ polypeptide" recited in claim 31 includes the substitutions recited in claims 45 and 46. Thus, claims 45 and 46 do not lack antecedent basis and the rejection should be withdrawn.

The Examiner also rejected claims 48 and 72 as allegedly being vague and ambiguous for reciting for terms related to "permeability", "PS product", "the protein", "different brain regions", and "adjustment of the reading after correction." The limitations of claims 48 and 72 pertain to characteristics of the claimed composition as detected by an assay that correlates with the unexpected results (i.e., enhanced permeability of a non-Aβ polypeptide across the blood brain barrier when linked to an Aβ polypeptide) of the therapeutic composition recited in the claims. Such an assay and the terms used in the claims are not only described in the specification as originally filed (see, e.g., page 1, lines 16-18; page 13, line 28 through page 14, line 27; and page 15, lines 13-20) but also were well known in the art as of the filing date of the present application. See, for example, Poduslo et al., Proc. Natl. Acad. Sci. USA, 89:2218-2222, 1992 and Poduslo et al., Neurobiol., 8:555, 567, 2004 (documents AW and AZ, respectively, cited in the Information Disclosure Statement). Accordingly, Applicants respectfully submit that one of skill in the art would not find claims 48 or 72 vague and/or ambiguous.

In view of the foregoing, Applicants request that the rejections under 35 U.S.C. § 112, second paragraph, be withdrawn.

III. The rejections under 35 U.S.C. § 102(b) should be withdrawn.

The Examiner rejected claims 31, 33-35, 43, 48, 69 and 72-73 under 35 U.S.C. § 102(b) as allegedly being anticipated by Solomon et al., (Proc. Natl. Acad. Sci. USA, 94:4109-4112, 1997; hereinafter "Solomon"). Applicants request reconsideration of the rejection in view of the following remarks.

Claims 31 and 73, and those claims directly or indirectly dependent therefrom, recite a composition comprising a ***sterile, pharmaceutically acceptable*** carrier or excipient. Contrary to the Examiner's assertion, Solomon did not clearly disclose or suggest that immunocomplexes of Aβ and anti-Aβ antibody should be isolated and placed in a composition with a *sterile, pharmaceutically acceptable* carrier or excipient. The Examiner points to pages 4109-4111 and Figure 5 of Solomon for disclosure of a sterile pharmaceutically acceptable carrier or excipient. Applicants have reviewed the sections noted by the Examiner and do not find such a specific disclosure or suggestion of a sterile, pharmaceutically acceptable carrier or excipient as required by claims 31 and 73.

Solomon described treating PC 12 cells cultured in a DMEM cell culture medium, containing components which the skilled artisan would recognize as inappropriate in a carrier or excipient for a therapeutic composition. These components, inappropriate for a pharmaceutically acceptable carrier or excipient, included penicillin, streptomycin, and insulin, any one of which is well known to cause adverse reactions or toxicity at least in some patients under certain conditions; plus pH indicator phenol red, which is reported to have estrogenic activity in estrogen-responsive cells (Berthois et al., Proc Natl Acad Sci U S A. 1986 April; 83(8): 2496–2500, appended hereto as Appendix B) and, at least during three hours of incubation for viability assays, the common toxicity indicator compound (and mutagen) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (see, Solomon et al., at page 4109, second column, last two paragraphs; see, also, Sigma, In vitro toxicology assay kit, appended as Exhibit C, at column 2 "Warning"). In some experiments fibrillar Aβ was added concomitantly with increasing concentrations of anti-Aβ antibodies into the culture medium, and immunocomplexes formed incidentally in the culture medium (see, Solomon et al. at page 4110, first column, lines 2-7), but the culture medium clearly did *not* constitute a sterile, *pharmaceutically acceptable* carrier or excipient, as recited in claims 31 and 73.

In some other experiments, as the Examiner noted in the middle of page 5 of the Office Action, soluble Aβ and mAb 6C6 were preincubated together before being added to the cells for viability testing (see, Solomon et al. at page 4110, first column, lines 7-14). However, at pages 4109-4111 and the caption of Fig. 5, Solomon et al. failed to clearly disclose what sort of medium or carrier containing the preincubated Aβ and mAb 6C6 was added to the cells in the culture medium in those experiments; it is unclear whether any inappropriate components for a pharmaceutically acceptable carrier or excipient may have been included. Consequently, Solomon et al. failed to clearly disclose a therapeutic composition comprising, inter alia, a *sterile, pharmaceutically acceptable* carrier or excipient. Accordingly, Solomon does not anticipate any of claims 31 and 73 and claims directly or indirectly dependent therefrom, because it fails to disclose each and every element of the claim.

Also, it would be improper to reject independent claims 31 and 73 and those claims directly or indirectly dependent therefrom because Solomon failed to disclose or suggest an Aβ polypeptide linked to a therapeutic non-Aβ polypeptide, wherein the non-Aβ polypeptide improves or stabilizes one or more clinical features of a CNS disorder in a patient, as recited in amended claim 31 or 73.

The Examiner further rejected claims 31, 33, 42, 43, 45, 46, 48, 67, 68 and 70-73 under 35 U.S.C. § 102(b) as allegedly being anticipated by Schenk. The Examiner asserted that because Schenk discloses compositions comprising Aβ conjugated to a molecule that promotes delivery of Aβ to the bloodstream of a patient and/or promotes an immune response against Aβ, Schenk anticipates the present claims. Applicants disagree.

Schenk does not anticipate the claimed invention because Schenk failed to disclose or suggest an Aβ polypeptide linked to a therapeutic non-Aβ polypeptide, wherein the ***non-Aβ polypeptide*** improves or stabilizes one or more clinical features of a CNS disorder in a patient, as recited in claim 31, 67 or 73. According to Schenk, at page 4, line 34-37, Aβ or an active fragment of Aβ can be linked to a fusion or conjugate polypeptide (i.e., the non-Aβ polypeptide), which is (1) a molecule that promotes delivery of Aβ to the bloodstream of a patient, and/or (2) a molecule that promotes an immune response against Aβ; Schenk provides examples of the conjugate, at page 5, lines 1-5, namely, cholera toxin, attenuated diphtheria toxin CRM197, or a [generic] "immunoglobulin." The Examiner has failed to

provide any evidence to establish that any of these molecules is inherently a therapeutic for a CNS disorder (i.e., improves or stabilizes one or more clinical features of a CNS disorder, as recited in claim 31, 67 or 73). As explained in MPEP §2112, "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is ***necessarily present*** in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'" *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted, emphasis added). "In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic *necessarily* flows from the teachings of the applied prior art." Ex parte Levy, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis in original).

Thus, Applicants respectfully submit that there is no proper case of anticipation with respect to claims 31, 67 and 73 and those claims directly or indirectly dependent thereon because there is no evidence that the conjugate or fusion polypeptides (i.e., the non-Aβ polypeptide) described in Schenk must necessarily improve or stabilize one or more clinical features of a CNS disorder in a patient as defined in the specification and in claims 31 and 73. In any event, a *prima facie* case of anticipation can be rebutted by evidence showing that the prior art products do not necessarily possess the characteristics of the claimed product. Such rebuttal evidence is provided by the disclosure of Schenk itself, which teaches that the conjugate or fusion polypeptides are for entirely different purposes than treating CNS disorders (i.e., for displaying the Aβ polypeptide, promoting delivery to the bloodstream, or promoting an immune response).

Applicants note that claim 73 is parallel to claim 31 but additionally recites a number of specific CNS disorders, in addition to the limitation that the non-Aβ polypeptide improves or stabilizes one or more clinical features of a CNS disorder in a patient. There is no evidence in the cited art that any of the conjugate or fusion polypeptides disclosed in Schenk is a therapeutic for the listed CNS disorders according to claim 73. In view of the foregoing, applicants respectfully request that the rejection of claims 31, 33, 42, 43, 45, 46, 48 and 70-73 under 35 U.S.C. §102(b) be withdrawn.

IV. The rejection under 35 U.S.C. § 103(a) should be withdrawn.

The Examiner rejected claims 36-40 and 44 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Solomon.

As discussed above in Section III, Solomon neither discloses or suggests that its composition comprises a sterile pharmaceutically-acceptable carrier or excipient or that the antibody in the immunocomplex improves or stabilizes one or more clinical features of a CNS disorder in a patient as defined in the specification and in claim 31, from which claims 36-40 and 44 depend. Moreover, Solomon does not disclose or suggest that a composition comprising an Aβ polypeptide linked to a non-Aβ polypeptide can be used to treat a CNS disorder. Because the cited art fails to disclose or suggest each and every element of the claims, Applicants respectfully submit that the Examiner has not established a *prima facie* case of obviousness. Accordingly, the rejection of claims 36-40 and 44 should be withdrawn.

V. Conclusion

It is believed that the foregoing responds to all of the Examiner's concerns. If the Examiner believes that a telephone conversation would expedite allowance of the claims, she is invited to contact the undersigned agent at the number below. The Director is hereby authorized to charge any additional fees associated with the filing of this paper to Deposit Account No. 13-2855, under order no. 01017/30016A.

Dated: May 22, 2008

Respectfully submitted,

By: /Jeanne M. Brashear/56,301
Jeanne M. Brashear
Registration No.: 56,301
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300
Agent for Applicants

APPENDIX A

degenerative disorders. The social and travel history provides information about exposure to HIV risk factors, environmental toxins, and infectious agents.

NEUROLOGIC EXAMINATION

The neurologic examination begins with careful observation of the patient during history taking. The speed, symmetry, and coordination needed for moving from a chair to the examining table, along with posture and gait, are noted. The patient's demeanor, dress, and responses yield information about mood and social adaptation. The patient's reliance on others to answer questions may indicate failing memory. Errors in language, speech, or praxis; neglect of space; unusual posturing; and other disorders of movement may be apparent before formal testing begins.

Guided by an initial formulation of the anatomy and pathophysiology of the lesion, the examiner expands some components of the examination and deletes others. For a less skilled observer, a full neurologic screening may help detect an unsuspected abnormality or confirm normal status.

Mental status examination (see also Ch. 185): A patient's ability to attend must be assessed first; an inattentive patient cannot be accurately assessed further. Any hint of cognitive decline should lead to administration of the complete Mini-Mental Status Examination (see FIG. 165–1), which tests multiple aspects of cognitive function. They include orientation to time, place, and person; memory; verbal and mathematical abilities; judgment; and reasoning. Loss of orientation to person occurs only in severely obtunded, delirious, or demented persons; as an isolated symptom, it suggests malingering. Insight into illness and fund of knowledge are also assessed, although some responses may be influenced by education. Affect and mood are evaluated (see Ch. 189).

Normally, a person should be able to follow a complex command that involves three body parts and discriminates between right and left (eg, "Put your right thumb on your left ear, and stick out your tongue"). Naming of simple objects and body parts, reading, writing, and repetition are assessed; if function is disturbed, other tests of aphasia ar performed (see Ch. 169). Spatial perceptio can be tested by asking the patient to imitat simple and complex finger constructions an to draw a clock, cube, house, or interlockin pentagons (see FIG. 165–1). The effort ex pended is often as informative as the fina product and may identify impersistence, per severation, micrographia, and hemispatia neglect. Praxis can be checked by asking th patient to use a toothbrush or comb or t take a match out of a box and strike it.

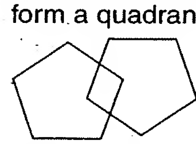
Cranial nerve examination: The exten of the cranial nerve examination depends o the site of the suspected lesion. Smell (1s [olfactory] cranial nerve) is generally no tested in patients with muscle disease bu should always be tested in those with sus pected lesions of the anterior fossa or afte head trauma. The patient is asked to identif odors (eg, soap, coffee, cloves) presented t each nostril. Alcohol, ammonia, and othe irritants test the nociceptive receptors of th 5th (trigeminal) cranial nerve, so they are no used except to detect a malingerer.

The 2nd (optic), 3rd (oculomotor), 4t (trochlear), and 6th (abducens) cranial nerves are tested as part of the visual system (see Chs. 101 and 178). Visual acuity (corrected for refractive error) is tested, visual fields are examined, and funduscopy is performed. The shape, size, reactivity to light, and accommodation of the pupils and extraocular eye movements are noted.

To test the 5th (trigeminal) nerve's three sensory divisions (ophthalmic, maxillary, and mandibular), an examiner uses a pin to evaluate facial sensation and brushes a wisp of cotton against the lower limbus of the cornea to evaluate the corneal reflex. If sensation in the face is lost, the angle of the jaw should be examined. This area, innervated by spinal root C-2, should be spared when the problem is an isolated trigeminal deficit. A weak blink due to facial weakness (eg, 7th cranial nerve paralysis) should be distinguished from a depressed corneal response. Patients who wear contact lenses often have reduced or absent corneal reflexes. Supranuclear corneal hyposensitivity associated with hypalgesia of body as well as face must be distinguished from peripheral lesions. Trigeminal motor function is tested by palpating the masseter muscles while the patient clenches his teeth and by asking him to open his jaw against resistance. The jaw will de-

	Score	*Points*
Orientation		
1. What is the		
Year?	____	1
Season?	____	1
Date?	____	1
Day?	____	1
Month?	____	1
2. Where are we		
State?	____	1
County?	____	1
Town/city?	____	1
Floor?	____	1
Address/name of building?	____	1
Registration		
3. Name three objects, taking one second to say each. Then ask the patient all three after you have said them. Repeat the answers until the patient learns all three.	____	3
Attention and Calculation		
4. Ask for serial sevens. Give one point for each correct answer. Stop after five answers. Alternative: Spell *world* backward.	____	5
Recall		
5. Ask for names of three objects learned in question 3. Give one point for each correct answer.	____	3

	Score	*Points*
Language		
6. Point to a pencil and a watch. Have the patient name them as you point.	____	2
7. Have the patient repeat "No ifs, ands, or buts."	____	1
8. Have the patient follow a three-stage command: "Take the paper in your right hand. Fold the paper in half. Put the paper on the floor."	____	3
9. Have the patient read and obey the following: "Close your eyes."	____	1
10. Have the patient write a sentence of his or her own choice. (The sentence should contain a subject and an object and should make sense. Ignore spelling errors when scoring.)	____	1
11. Enlarge the design printed below to 1 to 5 cm per side and have the patient copy it. (Give one point if all the sides and angles are preserved and if the intersecting sides form a quadrangle.)	____	1



Total	____	30

FIG. 165–1. Mini-Mental Status Examination form.

viate to the side of a weakened pterygoid muscle.

The 7th (facial) cranial nerve (see also FACIAL NERVE DISORDERS in Ch. 178) is tested by looking for hemifacial weakness. Asymmetry of facial movements is often more obvious during spontaneous conversation, especially when the patient smiles or, if obtunded, grimaces at a noxious stimulus. The examiner looks for a depressed nasolabial fold and widened palpebral fissure on the side of weakness. If furrowing of the forehead and eye closure are preserved, the cause of lower facial weakness is probably central rather than peripheral. Taste in the anterior 2/3 of the tongue can be tested with sweet, sour, salty, and bitter solutions placed on both sides of the tongue. Hyperacusis may be detected with a vibrating tuning fork held next to the ear.

The 8th (vestibulocochlear, acoustic) cranial nerve carries auditory and vestibular input, so hearing and balance must be evaluated (see Ch. 82 and ACOUSTIC NEUROMA in Ch. 85).

The 9th (glossopharyngeal) and 10th (vagus) cranial nerves are usually examined together. The palate should elevate symmetrically, and gag is elicited by touching each side of the posterior pharynx with a tongue blade. However, bilateral absence of the gag reflex is common and may not be significant. In an unresponsive, intubated patient, suctioning the endotracheal tube should trigger coughing. The vocal cords are inspected if hoarseness is evident. Isolated hoarseness (with normal gag and palatal elevation) should prompt a search for lesions compressing the recurrent laryngeal nerve (eg, mediastinal lymphoma, aortic aneurysm).

The 11th (spinal accessory) cranial nerve supplies the sternocleidomastoid and upper trapezius. The former is tested by having a patient turn his head against resistance supplied by the examiner's hand while the examiner palpates the active muscle (opposite the turned head). The trapezius is tested by elevating the shoulders against resistance supplied by the examiner.

The 12th (hypoglossal) cranial nerve innervates the tongue, which is inspected for atrophy, fasciculations, and weakness (deviation toward the side of the lesion).

An abnormality of one cranial nerve requires meticulous scrutiny of adjacent nerves. Such distinctions can be urgent, for example, when brain stem ischemia must be distinguished from a rapidly expanding aneurysm producing cranial nerve paralysis.

Examination of the motor system: The limbs and shoulder girdle should be fully exposed, then inspected and palpated for atrophy, hypertrophy, fasciculations, other involuntary movements (eg, chorea, athetosis, myoclonus, tremor), and asymmetric development. Passive flexion and extension of the limbs in a relaxed patient provide information about muscle tone. Decreased muscle bulk indicates atrophy, but bilateral atrophy or atrophy in large or concealed muscles (unless advanced) may not be obvious. In older persons, loss of some muscle is common (sarcopenia). Hypertrophy occurs when one muscle works harder, substituting for another; pseudohypertrophy occurs when muscle tissue is replaced by excessive fibrous tissue or a storage material.

Fasciculations, the most common abnormal movement, are brief, fine, irregular twitches of the muscle visible under the skin. They usually indicate lesions of the lower motor neuron (eg, nerve degeneration or injury and regeneration) but sometimes occur in normal muscle, particularly in the calf muscles of older persons. **Myotonia,** the decreased relaxation of muscle after a sustained contraction or direct percussion of the muscle, occurs in myotonic dystrophy and may cause disability (eg, inability to relax and quickly open the closed hand). **Increased resistance followed by relaxation** (clasp-knife rigidity or phenomenon) occurs with upper motor neuron lesions. Basal ganglia disorders usually produce cogwheel rigidity.

Assessment of muscle strength: For patients, weakness has various meanings, such as fatigue, clumsiness, or numbness. A complaint of muscle weakness should be characterized precisely by describing the exact location, time of occurrence, precipitating and ameliorating factors, and associated symptoms and signs. The patient extends his arms, then legs, to be inspected for weakness (a weak limb soon begins to drift downward), tremor, and other involuntary movements. Strength of specific muscle groups may be tested against resistance. Pain in a muscle or affected joint may preclude an active contraction. With hysterical weakness, resistance to movement may be normal, followed by a sudden giving way.

TABLE 171–1. DIFFERENCES BETWEEN DELIRIUM AND DEMENTIA*

Delirium	Dementia
Develops rapidly	Develops slowly
Fluctuating course	Slowly progressive course
Potentially reversible	Not reversible
Profoundly affects attention	Profoundly affects memory
Focal cognitive deficits	Global cognitive deficits
Usually caused by systemic medical disease or drugs	Usually caused by Alzheimer's disease or cerebrovascular disease (multi-infarct dementia)
Requires immediate medical evaluation and treatment	Requires nonemergency medical evaluation and treatment

*These differences generally are true and are helpful diagnostically, but exceptions are not rare. For example, traumatic brain injury occurs suddenly but may result in severe, permanent dementia; hypothyroidism may produce the slowly progressive picture of dementia but may be completely reversible with treatment.

Knowledge of baseline function is essential for determining the extent and rate of change.

Of greatest clinical importance is avoiding the common clinical error of mistaking delirium for dementia in a sick older patient. The evaluation of dementia can be slow and prolonged because the cause is rarely immediately life-threatening. However, because delirium is usually caused by an acute illness or drug toxicity, patients with it may worsen rapidly and are at risk of death unless they are quickly diagnosed and treated.

DELIRIUM

(Acute Confusional State)

A clinical state characterized by fluctuating disturbances in cognition, mood, attention, arousal, and self-awareness, which arises acutely, either without prior intellectual impairment or superimposed on chronic intellectual impairment.

Some practitioners use the terms delirium and acute confusional state synonymously; others use delirium to refer to a subset of confused people with hyperactivity. Still others use delirium to refer to full-blown confusion and confusional state to refer to mild disorientation.

A person who is less alert (with clouding of consciousness) and has difficulty paying attention also has difficulty accurately perceiving and interpreting data from the environment and acquiring or remembering new information; he may misinterpret factual information or have illusions. As a result, the person does not reason logically, has difficulty manipulating symbolic data (eg, performing arithmetic or explaining proverbs), becomes anxious and agitated or withdraws from the environment, and may think in paranoid and delusional ways.

Etiology

Delirium may occur in persons with a normal brain but is more common in those with underlying brain disease, such as dementia. It is more common in the elderly, probably due to changes in neurotransmitters, cerebral cell loss, and concomitant disease. Delirium may be due to primary brain diseases or diseases elsewhere in the body that affect the brain; causes are usually metabolic, toxic, structural, or infectious. Regardless of cause, the cerebral hemispheres or the arousal mechanisms of the thalamus and reticular activating system of the brain stem become physiologically impaired. Disruption of sleep and extreme stress superimposed on acute disease may worsen symptoms of delirium (as in intensive care psychosis).

Metabolic or toxic causes: Virtually any metabolic disorder can cause delirium. Some important metabolic and toxic causes of delirium are listed in TABLE 171–2. In elderly persons, drug side effects are the most common cause.

Structural causes: Structural lesions that can precipitate delirium include vascular occlusion and cerebral infarction, subarachnoid hemorrhage, cerebral hemorrhage, primary or metastatic brain tumors, subdural hematomas, and brain abscesses. Most structural lesions can be detected by CT or MRI, and many produce focal neurologic signs.

Infectious causes: Delirium may be caused by acute meningitis or encephalitis or by infections outside the brain, perhaps through the elaboration of toxins or production of fever. Pneumonia (even without im-

TABLE 171–2. METABOLIC AND TOXIC CAUSES OF DELIRIUM

Disorders	Drugs With Anticholinergic Properties	Other Drugs
Anoxia	Antiemetics	Alcohol
Hyperkalemia	Antihistamines (eg, diphenhydramine)	Antihypertensives
Hyperparathyroidism	Antiparkinsonian drugs	Benzodiazepines
Hyperthyroidism	Antipsychotics	Cimetidine
Hypoglycemia	Antispasmodics	Digoxin
Hypokalemia	Muscle relaxants	Narcotics
Hypothyroidism	Tricyclic antidepressants	Other CNS depressants
Metabolic acidosis		
Postconcussion		
Postictal state		
Transient ischemia		

paired oxygenation), urinary tract infections, sepsis, or fever from viral infections can produce confusion in the vulnerable brain. Slower-developing embolic abscesses or opportunistic infections are difficult to diagnose clinically and, in some cases, require brain biopsies for proper evaluation.

Symptoms and Signs

The symptoms of delirium often fluctuate rapidly, even within a matter of minutes, and tend to be worse late in the day (sundowning). The most prominent is a clouding of consciousness accompanied by disorientation to time, place, or person. The ability to pay attention is poor. Confusion regarding day-to-day events and daily routines is common. Changes in personality and affect are common. Symptoms include irritability, inappropriate behavior, fearfulness, excessive energy, or even frankly psychotic features, such as delusions, hallucinations (commonly visual), or paranoia. Some persons become quiet, withdrawn, or apathetic, whereas others become agitated or hyperactive; physical restlessness is often expressed by pacing. A person may display contradictory emotions within a short time span. Thinking becomes disorganized, and speech is often disordered, with prominent slurring, rapidity, neologisms, aphasic errors, or chaotic patterns. Normal patterns of sleeping and eating are usually grossly distorted. Some persons experience dizziness.

Diagnosis

A rapid medical evaluation is imperative because delirium can have a grave prognosis and the underlying condition is often treatable. According to some estimates, 18% of hospitalized elderly persons with delirium die, and hospitalization is twice as long for those who develop confusion as for those who do not.

The diagnosis rests almost entirely on clinical grounds. Diagnostic criteria are listed in Table 171–3. Laboratory tests should include full chemistries, CBC with differential, a test for syphilis such as the Venereal Disease Research Laboratories (VDRL) test, urinalysis with culture, blood cultures, thyroid function tests, vitamin B_{12} levels, and a toxicology screening. Unless status epilepticus (an extremely rare finding in the elderly) or encephalitis is suspected, EEGs, lumbar punctures, single photon emission computed tomography, and positron emission tomography are not useful. A single CT scan with contrast can detect old or recent infarctions or subdural hematomas.

Delirium with apathy must be differentiated from depression, especially in the elderly, although the two often occur together. Similarly, agitation and hallucinations associated with delirium must be distinguished from those of a functional psychosis—a psychiatric disorder that almost always lacks the disorientation, memory loss, and cognitive impairment found in delirious (or intoxicated) patients. A history of manic illness or schizophreniform disorders suggests a diagnosis of psychiatric disease.

Systemic medical diseases may precipitate delirium and should be sought to guide treatment; an example is the Wernicke-Korsakoff syndrome, which is marked by confusion, disorientation, and memory loss. Hypothermia, tachycardia, hypotension, tremor, and ophthalmoplegia strongly suggest alcohol-related disease. Status epilepti-

TABLE 171–3. DIAGNOSTIC CRITERIA FOR DELIRIUM

Disturbance of consciousness (ie, reduced clarity of awareness of the environment) with reduced ability to focus, sustain, or shift attention.

Change in cognition (eg, memory deficit, disorientation, language disturbance) or development of a perceptual disturbance that is not better accounted for by preexisting, established, or evolving dementia.

The disturbance develops over a short time (usually hours to days) and tends to fluctuate during the course of the day.

For delirium due to a general medical condition:
- Evidence from the history, physical examination, or laboratory tests that the disturbance is due to the direct physiologic consequences of a general medical condition.

For substance intoxication delirium:
- Evidence from the history, physical examination, or laboratory tests that either
  1. Symptoms listed in the first two criteria developed during substance intoxication
  2. Drug use is etiologically related to the disturbance.

For substance withdrawal delirium:
- Evidence from the history, physical examination, or laboratory tests that the symptoms listed in the first two criteria developed during or shortly after a withdrawal syndrome.

For delirium due to multiple etiologies:
- Evidence from the history, physical examination, or laboratory tests that the delirium has more than one etiology (eg, more than one general medical condition, a general medical condition plus substance intoxication or a drug side effect).

Modified from American Psychiatric Association: *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition. Washington, DC, American Psychiatric Association, 1994, pp. 129, 131–133; reprinted with permission. Copyright, 1994 American Psychiatric Association.

cus consisting of absence or complex partial seizures can produce a confused state that is hard to distinguish from delirium. The seizure states, however, produce a steadier but less intense pattern of bewilderment and less drowsiness than does delirium. Despite a confused appearance, affected epileptic patients usually have a surprisingly good sense of direction compared with most delirious patients. Nonconvulsive status epilepticus can be readily detected by EEG. EEG recordings with spike and wave or sharp wave discharges are diagnostic. Delirium alone seldom precipitates convulsive status epilepticus, but a generalized tonic-clonic seizure often results in a state of delirium for up to a day or more. In encephalopathy, the EEG shows a rhythm slower than alpha from both hemispheres. Triphasic waves may appear in hepatic or renal encephalopathy.

Treatment

Symptoms are usually reversible when the underlying cause is identified quickly and managed properly, particularly if the cause is hypoglycemia, an infection, an iatrogenic factor, drug toxicity, or an electrolyte imbalance. However, recovery may be slow (days to even weeks or months), especially in the elderly.

All unnecessary drugs should be stopped. Identifiable disease should be treated, and fluids and nutrients should be given. A patient suspected of alcohol abuse or withdrawal should be given thiamine 100 mg IM daily for at least 5 days, to ensure absorption. During hospitalization, such patients should be monitored for signs of withdrawal, which can be manifested by autonomic disturbances and worsening confusion.

The environment should be as quiet and calm as possible, preferably with low lighting but not total darkness. Staff and family members should reassure the patient, reinforce orientation, and explain proceedings at every opportunity. Additional drugs should be avoided unless needed to reverse the underlying condition. However, sometimes agitation must be treated symptomatically, particularly when it threatens the well-being of the patient, a caregiver, or a staff member. Restraints used judiciously can help prevent the patient from pulling out IV and other lines. Restraints should be applied by someone trained in their use, released at least every 2 h to prevent injury, and discontinued as soon as possible.

Few data are available to guide the choice of drugs to treat delirium. Low doses of haloperidol (as little as 0.25 mg po, IM, or IV) or thioridazine (5 mg po) can help in managing the delirious patient. Larger doses

(haloperidol 2 to 5 mg or thioridazine 10 to 20 mg) are sometimes needed. Newer drugs, such as risperidone, can be used instead of haloperidol for oral therapy but are not available IM or IV. Short- or intermediate-acting benzodiazepines (eg, alprazolam, triazolam) can control agitation over the short term; benzodiazepines may worsen confusion, but if required, the smallest effective dose should be used. All psychoactive drugs should be reduced and then eliminated as soon as possible so that recovery can be assessed.

DEMENTIA

A chronic deterioration of intellectual function and other cognitive skills severe enough to interfere with the ability to perform activities of daily living.

TABLE 171–4 lists many of the known causes. Dementia may occur at any age and can affect young people as the result of injury or hypoxia. However, it is mostly a disease of the elderly, affecting > 15% of persons > 65 yr old and as many as 40% of persons > 80 yr old. It accounts for more than half of nursing home admissions and is the condition most feared by aging adults.

Although new memory retention decreases with age, other cognitive functions remain relatively intact. Therefore, dementia is a marked change from normal functioning. Traditionally, dementia has been classified as Alzheimer's or non-Alzheimer's type. Dementia is sometimes categorized as reversible or irreversible according to its cause, although this complicates the distinction between dementia and delirium.

In elderly patients, the clinician should differentiate the early-stage cognitive deficit of dementia from **age-associated memory impairment.** Persons with age-associated memory impairment have a relative deficiency in recall compared with others their age. They tend to learn new information slowly; if they are given extra time for such tasks, their intellectual performance is usually adequate.

TABLE 171–4. CAUSES OF DEMENTIA

Metabolic-Toxic	Structural	Infectious
Anoxia	Alzheimer's disease	Bacterial endocarditis
B_{12} deficiency	Amyotrophic lateral sclerosis	Brain tumors (selective)
Chronic drug-alcohol-nutritional abuse	Brain trauma (acute severe)	Creutzfeldt-Jakob disease
Folic acid deficiency (?)	Chronic subdural hematoma	Gerstmann-Sträussler-Scheinker disease
Hypercalcemia associated with hyperparathyroidism	Dementia pugilistica	HIV-related disorders
Hypoglycemia	Brain tumor	Neurosyphilis (general paresis)
Hypothyroidism	Cerebellar degeneration	Tuberculous and fungal meningitis
Organ system failure	Communicating hydrocephalus	Viral encephalitis
Hepatic encephalopathy	Huntington's disease (chorea)	
Respiratory encephalopathy	Irradiation to frontal lobes	
Uremic encephalopathy	Multiple sclerosis	
Pellagra	Normal-pressure hydrocephalus	
	Parkinson's disease	
	Pick's disease	
	Progressive multifocal leukoencephalopathy	
	Progressive supranuclear palsy	
	Surgery	
	Vascular disease	
	Multi-infarct dementia	
	Wilson's disease	

(?) = uncertain.

TABLE 171–5. DIAGNOSTIC CRITERIA FOR DEMENTIA

Development of multiple cognitive deficits manifested by both:

1. Memory impairment (impaired ability to learn new information or to recall previously learned information)
2. One (or more) of the following cognitive disturbances:
   a. Aphasia (language disturbance)
   b. Apraxia (impaired ability to carry out motor activities despite intact motor function)
   c. Agnosia (failure to recognize or identify objects despite intact sensory function)
   d. Disturbance in executive functioning (ie, planning, organizing, sequencing, abstracting).

Each of the cognitive deficits described above causes significant impairment of social or occupational functioning and represents a significant decline from a previous level of functioning.

The course is characterized by gradual onset and continuing cognitive decline.

Deficits do not occur exclusively during the course of delirium.

For Alzheimer's disease:

The cognitive deficits listed in the first criterion (parts 1 and 2) are not due to any of the following:

1. Other CNS conditions that cause progressive deficits in memory and cognition (eg, cerebrovascular disease, Parkinson's disease, Huntington's disease, subdural hematoma, normal-pressure hydrocephalus, brain tumor)
2. Systemic conditions known to cause dementia (eg, hypothyroidism, vitamin B_{12} or folic acid deficiency, niacin deficiency, hypercalcemia, neurosyphilis, HIV infection)
3. Substance-induced conditions.

For vascular dementia:

Focal neurologic signs and symptoms (eg, exaggeration of deep tendon reflexes, extensor plantar response, pseudobulbar palsy, gait abnormalities, weakness of an extremity) or laboratory evidence indicates cerebrovascular disease (eg, multiple infarctions affecting the cortex and underlying white matter) that is judged to be etiologically related to the disturbance.

For dementia due to other medical conditions:

Evidence from the history, physical examination, or laboratory tests indicates that the disturbance is the direct physiologic consequence of such conditions as Parkinson's disease, Huntington's disease, Pick's disease, Creutzfeldt-Jakob disease, head trauma, HIV infection, normal-pressure hydrocephalus, hypothyroidism, brain tumor, vitamin B_{12} deficiency, or intracranial radiation.

Modified from American Psychiatric Association: *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition. Washington, DC, American Psychiatric Association, 1994, pp. 142–143, 146, 152; reprinted with permission.

Dementia of depression (formerly termed pseudodementia) is usually used to describe patients who may appear demented at first but have depression rather than a neuropathologic disorder. They regain mental competence when depression is treated. More commonly, depression and dementia coexist; in such cases, treating depression is still important but does not fully restore cognition.

The diagnosis of dementia is based on a thorough history and mental status examination. Diagnostic criteria are listed in TABLE 171–5. Identification of drugs or other toxic factors may require the assistance of family members. Depressed patients can be distinguished because they eat little, are constipated, sleep less than normal, and behave best at night. They respond slowly but often accurately; they may be semimute, but very few are aphasic. They rarely forget major current events or matters of great personal importance. Severely depressed patients tend to complain of memory loss disproportionate to their examination results. In contrast, patients with dementia seldom complain of memory problems. In contrast to demented patients, depressed patients have unremarkable neurologic examinations.

Prognosis and Treatment

The progression rate of dementia varies widely and depends on the cause. Dementia can be static if it follows acute severe brain injury due to trauma or transient asystole. Abstention from alcohol by patients with alcoholic dementia can lead to substantial long-term improvement. Controlling hyper-

tension or diabetes may slow or arrest the progression of vascular (multi-infarct) dementia, resulting in improvement in a few patients.

Even when intellectual function cannot be restored or its decline arrested, simple supportive measures (eg, frequent orientation reinforcement; a bright, cheerful, familiar environment; a minimum of new stimulation; regular low-stress activities) can help greatly. Orientation to time is helped by using large calendars and clocks and routinizing daily activities; orientation to person is helped by medical staff members wearing large name tags and repeatedly introducing themselves. The patient requires time to adjust to and become familiar with new surroundings, routines, and people. Explanations should be precise and simple, and nonessential procedures omitted.

Quiet, dark private rooms should be avoided. The room should be reasonably bright and contain sensory stimuli, such as a night-light and a radio or television, to help the patient remain oriented and to focus his attention. The environment should also be safe and secure; for example, signal systems can be installed to monitor those who tend to wander. Overstimulation and understimulation should be avoided. Familiar people and frequent visits by staff members encourage the patient to remain social; isolation should be avoided. Staff members should avoid confronting or physically intimidating the patient. The patient should remain as active as possible; families should include him in activities but avoid activities that cause anxiety or confusion. Exercise to reduce restlessness, improve balance, and maintain cardiovascular tone should be performed daily. Occupational and music therapy helps maintain fine motor control and provides nonverbal stimulation. Group therapy (reminiscence therapy and socialization activities) may help conversational and interpersonal skills, and family counseling can teach family members how to prevent the patient from falling and avoid being hit by him during periods of agitation. If daily routines can be simplified and the caregivers' expectations reduced without the patient sensing a total loss of self-control or personal dignity, the patient may actually show some improvement.

Functioning can often be further improved by eliminating or strictly limiting drugs with CNS activity. The optimal use of psychoactive drugs in the elderly to control unwanted behaviors is controversial. However, antidepressants can temporarily improve function in patients who develop clinical depression. Depression should be treated with nonanticholinergic antidepressants, and anxiety and sleep disorders may be treated with judicious doses of short- or medium-acting benzodiazepines. Other behaviors are more problematic. Antipsychotic drugs are commonly used, but their effectiveness has not been established except in psychotic patients. Toxicity occurs frequently and can be severe. If used at all, doses should be kept very small, and they should not be used for long periods. There is no evidence that cholinergic-enhancing drugs benefit patients with non-Alzheimer's dementias.

After the medical evaluation is completed and a course of treatment is established, most of the responsibility falls on the family. Although a cure is rarely available, the clinician can still help the family, eg, by helping them understand that although the disease is progressive in nature, many complicating factors can be controlled. The stresses of caring for a person with dementia are tremendous and can adversely affect the physical and emotional health of family members, compromising care. The clinician can recognize the early symptoms of caregiver burnout and guide families to the appropriate social agencies, thereby enhancing the patient's overall care. Team members (social worker, nutritionist, nurse, home health aide, and others) can assist in providing counseling and support to patients and their caregivers.

Disability may become so severe that aggressive—or indeed any—treatment of other diseases is no longer warranted (see Ch. 294), and death may follow pneumonia or another acute illness. The patient's wishes about care should be clarified before he is incapacitated. Financial and legal arrangements (eg, durable power of attorney, durable power of attorney for health care) should be made in the early stage of the disease.

ALZHEIMER'S DISEASE

A progressive, inexorable loss of cognitive function associated with an excessive number of senile plaques in the cerebral cortex and subcortical gray matter,

which also contains β-amyloid and neurofibrillary tangles consisting of tau protein.

Epidemiology

Early-onset forms account for only 2 to 7% of cases and are usually due to an inherited genetic mutation. The common form affects persons > 60 yr old, and its incidence increases as age advances.

Four million Americans have Alzheimer's disease, at an annual cost of about $90 billion, including medical and nursing home care, social services, lost productivity, and early death. The disease is about twice as common in women as in men (perhaps because women live longer, but female sex may be a risk factor). It accounts for > 65% of the dementias in the elderly. Vascular dementia and Alzheimer's disease coexist in about 15% of cases.

Etiology

The cause of Alzheimer's disease is not known. The disease runs in families in about 15 to 20% of cases. The remaining, so-called sporadic cases have some genetic determinants. At least four distinct genes, located on chromosomes 1, 14, 19, and 21, influence initiation and progression. Chromosome 21 generates the precursor protein for the amyloid protein, which accumulates in the brain of patients with Alzheimer's disease (as well as with other conditions). Chromosome 19 generates apolipoprotein (apo) E alleles 1 to 4 (ε1 to ε4). The presence of the ε4 allele increases the risk for Alzheimer's disease in whites; ε2 and ε4 alleles increase the risk in blacks. Trisomy 21 produces early Alzheimer's disease in persons with Down syndrome. These findings support the epidemiologic observation that the disease has an autosomal dominant genetic pattern in most early-onset and some late-onset cases but a variable late-life penetrance. Environmental factors are the focus of active investigation. Unproven speculations include low hormone levels and exposure to metals.

Pathogenesis

Neurons are lost within the cerebral cortex, hippocampus, and subcortical structures (including selective cell loss in the nucleus basalis of Meynert), locus caeruleus, and nucleus raphae dorsalis. Cerebral glucose use and perfusion is reduced in some areas of the brain (parietal lobe and temporal cortices in early-stage disease, prefrontal cortex in late-stage disease), as determined by positron emission tomography; whether this reduction precedes or follows cell death is not known. The microvasculature may also be affected, as seen in congophilic angiopathy.

Neuritic or senile plaques (composed of neurites, astrocytes, and glial cells around an amyloid core) and neurofibrillary tangles (composed of paired helical filaments) play a role in the pathogenesis of Alzheimer's disease. Senile plaques and neurofibrillary tangles occur with normal aging, but they are much more prevalent in persons with Alzheimer's disease.

Specific protein abnormalities occur in Alzheimer's disease. β-amyloid protein is thought to contribute to the pathogenesis of the disease. Ongoing research is trying to determine if amyloid is a toxic cause of cognitive decline or a biologic reaction and secondary phenomenon. Apo E proteins, produced in the brain and liver, influence a number of cerebral processes, including amyloid deposition, cytoskeletal integrity, and efficiency of neuronal repair. Apo E's role in Alzheimer's disease is becoming more certain. The protein has three allelic forms called ε2, ε3, and ε4, resulting in six genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4. The risk of Alzheimer's disease is substantially increased in persons with two ε4 alleles, who are more likely to develop the disease between the ages of 60 and 75 yr. Incidence may be decreased in those who have the ε2 allele. Because about 40% of persons who reach age 85 yr develop some form of diagnosable dementia regardless of apo E status, this genetic test is not very useful in predicting whether a person will develop Alzheimer's disease later in life. The test is commercially available. Its usefulness as an adjunctive diagnostic test (rather than a predictive test) for Alzheimer's disease is under study.

Several proteins are abnormally increased in the brain and appear in the CSF. Whether they are causative or are markers for the disease is not certain. The tau protein (of neurofibrillary origin) has high specificity but low sensitivity for identifying a dementia as Alzheimer's disease; a slightly different type of tau protein also accumulates in patients with progressive supranuclear palsy

(see Ch. 179). Choline acetyltransferase is markedly reduced, decreasing the availability of acetylcholine. Somatostatin, corticotropin-releasing factor, and other neurotransmitters are also significantly reduced.

Symptoms and Signs

Alzheimer's disease can be divided into clinical stages. However, patients vary greatly, and disease progression often is not as orderly as the following description implies. The disease progresses gradually, although sometimes symptoms seem to plateau for a time.

The **early stage** is characterized by loss of recent memory, inability to learn and retain new information, language problems (especially word finding), mood swings, and personality changes. Patients may have progressive difficulty performing activities of daily living (eg, balancing their checkbook, finding their way around, or remembering where they put things). Abstract thinking or proper judgment may be diminished. Patients may respond to loss of control and memory with irritability, hostility, and agitation. Some patients have isolated aphasia or visuospatial difficulties. Although the early stage may not compromise sociability, families may report strange behavior (eg, the patient gets lost on the way to the store or forgets the name of a recent dinner guest), accompanied by the onset of emotional lability.

In the **intermediate stage,** patients become unable to learn and recall new information. Memory of remote events is affected but not totally lost. Patients may require assistance with bathing, eating, dressing, or toileting. Behavioral disorganization may be characterized by wandering, agitation, hostility, uncooperativeness, or physical aggressiveness. By this stage, patients have lost all sense of time and place because normal environmental and social cues are used ineffectively. Patients often get lost, sometimes to the point of being unable to find their own bedroom or bathroom. Although they remain ambulatory, they are at risk for falls or accidents secondary to confusion.

In the **severe stage,** patients are unable to walk or to perform any activity of daily living and usually are totally incontinent. Recent and remote memory is completely lost. Patients may be unable to swallow and eat and are at risk of malnutrition, pneumonia (especially from aspiration), and pressure sores. Placement in a long-term care facility often becomes necessary because they are totally dependent on others for care. Eventually, patients become mute. Because such patients cannot relate any symptoms to the physician and because elderly patients often have no febrile or leukocytic response to infection, the physician must rely on experience and acumen whenever a patient looks ill.

Motor or other focal neurologic features occur very late in the disease, although the incidence of seizures is somewhat increased at all stages. The **end stage** of Alzheimer's disease is coma and death, usually from infection.

Complications

Behavioral complications include hostility, agitation, wandering, and uncooperativeness. Psychiatric complications include depression, anxiety, and paranoid reactions. True psychosis (paranoia, delusions, and hallucinations) probably occurs in about 10% of patients with Alzheimer's disease. In addition, perhaps 80% of family members or caregivers develop depression over time. Metabolic problems (eg, dehydration, infection, drug toxicity) can worsen cognitive impairment and make patient management more difficult. Other complications include falls, incontinence, and confusion at dusk (sundowning). The drugs commonly used to treat Alzheimer's disease (especially antipsychotics for behavior disorders) can cause a parkinsonian movement disorder and orthostatic hypotension. Tricyclic drugs with anticholinergic side effects can cause constipation, urinary retention, glaucoma, and seizures. Nonprescription antihistamines can lead to worsened confusion. These complications put the patient at risk of premature institutionalization and should be avoided or quickly treated, because many can be controlled or reversed.

Diagnosis

The diagnosis is usually based on the history, physical examination, laboratory tests, and the exclusion of other causes of dementia. A formal mental status examination should be performed; the Folstein Mini-Mental Status Examination (see Fig. 165–1) is most commonly used. The Barthel scale can be used to assess activities of daily living.

For about 85% of patients with Alzheimer's disease, a correct diagnosis can be made on the basis of a thorough history and results of a standard neurologic physical examination. A brain tissue biopsy is rarely performed or useful.

The essential features of dementia are impairment of short-term memory and long-term memory, abstract thinking, and judgment; other disturbances of higher cortical function; and personality change. Progression of cognitive impairment confirms the diagnosis, and patients with Alzheimer's disease do not improve. The following criteria help establish a probable diagnosis of Alzheimer's disease: dementia established by clinical examination and documented by a formal test of mental status; deficits in two or more areas of cognition; progressive worsening of memory and other cognitive functions; no disturbance of consciousness; onset between ages 40 and 90 yr, most often after age 65; and no systemic or brain disorders that could account for the progressive deficits in memory and cognition. Assessment tools, such as the Hachinski Ischemic Score, can be used to differentiate vascular dementia (see below) from Alzheimer's disease.

The basic evaluation should include a CBC, electrolyte panel measurements, SMA-12/60 (Sequential Multiple Analyzer) tests, thyroid function tests, folate and vitamin B_{12} levels, VDRL test, and urinalysis; ECG and chest x-ray may be useful in some patients. If the history suggests a mass, if focal neurologic signs exist, or if the dementia is of brief duration, CT or MRI should be performed to rule out tumors, infarcts, subdural hematoma, and normal-pressure hydrocephalus. Positron emission tomography is primarily a research technique; however, simple photon emission tomography provides similar information about cerebral perfusion patterns and can contribute to the differential diagnosis in some cases. Lumbar puncture is rarely needed but should be considered if a chronic infection or neurosyphilis is suspected as the cause of cognitive impairment.

Depression, the most common psychiatric problem in the elderly, can closely mimic early-stage Alzheimer's disease and coexists in about 20% of cases; therefore, depression should be considered in patients who present with cognitive impairment.

Prognosis and Treatment

Cognitive decline is inevitable, but the rate of progression is unpredictable. Survival ranges from 2 to 20 yr, with an average of 7 yr.

General treatment principles for Alzheimer's disease are the same as those for all dementias (see Treatment under DEMENTIA, above).

Some drugs that enhance cholinergic neurotransmission, such as donepezil, can at least temporarily improve memory during the early stages of Alzheimer's disease. However, they do not modify the steady worsening of the underlying pathology. Tacrine produces more unwanted side effects. A trial of donepezil starting with 5 mg once daily in the evening and, after 4 to 6 wk, increasing to 10 mg may be considered; it should be continued for several months to assess effectiveness. Antioxidants (eg, vitamin E), estrogen therapy, and NSAIDs are under study.

Many drugs adversely affect the CNS, increasing confusion and lethargy. Sedatives, such as benzodiazepines, should be avoided when possible. Anticholinergic drugs, such as some tricyclic antidepressants, antihistamines, antipsychotics, and benztropine, should be avoided.

An extract of *Ginkgo biloba* called EGb may slow down or modestly reverse memory loss and other symptoms in patients with Alzheimer's disease or vascular dementia. The extract may act as a free-radical scavenger. Complications appear to be minor, but further studies are needed.

NON-ALZHEIMER'S DEMENTIAS

Lewy body dementia may be the second most common dementia after Alzheimer's disease. Lewy bodies are hallmark lesions of degenerating neurons in Parkinson's disease and occur in dementia with or without features of Parkinson's disease. In Lewy body dementia, Lewy bodies may predominate markedly or be intermixed with classic pathologic changes of Alzheimer's disease. Symptoms, signs, and course of Lewy body dementia resemble those of Alzheimer's disease, except hallucinations (mainly visual) are more common and patients appear to have an exquisite sensitivity to antipsychotic-induced extrapyramidal adverse effects.

APPENDIX B

Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2496–2500, April 1986
Cell Biology

Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture

(cell proliferation/human breast cancer/antiestrogens/hormone responsiveness/estrogen receptor)

YOLANDE BERTHOIS*†, JOHN A. KATZENELLENBOGEN‡, AND BENITA S. KATZENELLENBOGEN*§

Departments of *Physiology and Biophysics and ‡Chemistry, University of Illinois, and University of Illinois College of Medicine, Urbana, IL 61801

Communicated by Elwood V. Jensen, December 18, 1985

ABSTRACT **Although much attention has been paid to the removal of hormones from sera and to the development of serum-free media for studies on hormone-responsive cells in culture, little consideration has been given to the possibility that the media components themselves may have hormonal activity. We have found that phenol red, which bears a structural resemblance to some nonsteroidal estrogens and which is used ubiquitously as a pH indicator in tissue culture media, has significant estrogenic activity at the concentrations (15–45 μM) at which it is found in tissue culture media. Phenol red binds to the estrogen receptor of MCF-7 human breast cancer cells with an affinity 0.001% that of estradiol ($K_d = 2 \times 10^{-5}$ M). It stimulates the proliferation of estrogen receptor-positive MCF-7 breast cancer cells in a dose-dependent manner but has no effect on the growth of estrogen receptor-negative MDA-MB-231 breast cancer cells. At the concentrations present in tissue culture media, phenol red causes partial estrogenic stimulation, increasing cell number to 200% and progesterone receptor content to 300% of that found for cells grown in phenol red-free media, thereby reducing the degree to which exogenous estrogen is able to stimulate responses. The antiestrogens tamoxifen and hydroxytamoxifen inhibit cell proliferation below the control level only when cells are grown in the presence of phenol red; in the absence of phenol red, the antiestrogens do not suppress growth. The estrogenic activity of phenol red should be considered in any studies that utilize estrogen-responsive cells in culture.**

There has been a great interest in understanding the mechanisms by which hormones affect cell proliferation and protein synthesis. Cell culture systems have played a prominent role in these analyses, since they enable responses to be monitored under carefully controlled conditions of hormone exposure (1, 2). Of the sex steroid hormones, estrogens are well known to stimulate a variety of biosynthetic processes in hormone-responsive target cells, such as those of the breast and uterus (3–5).

In studies evaluating hormone action in cultured cells, researchers have gone to great lengths to eliminate sources of estrogens from sera used in cell cultures so that they have cells in an unstimulated state (6–8), and considerable efforts also have been applied towards the development of serum-free media (9, 10). However, little attention has been paid to components of the cell culture media themselves that might have hormonal activity.

In our studies aimed at understanding estrogen and antiestrogen action in estrogen-responsive cells, we have been struck by the curious observation that antiestrogens suppress growth below that of control cells in the apparent absence of estrogens (6, 11), suggesting that this growth suppression might be mediated by an estrogen-noncompetitive process (11–14) or that cells in the control media might be inadvertently exposed to an estrogenic stimulus.

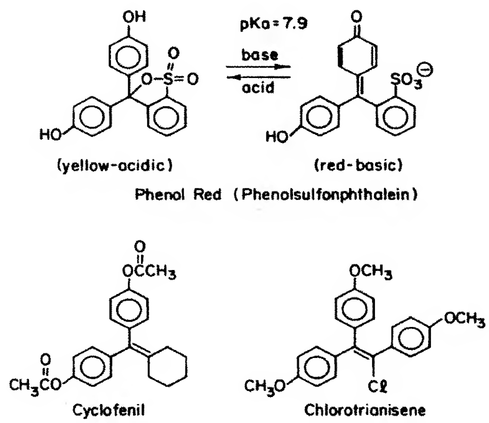


FIG. 1. Structure of phenol red and some structurally related nonsteroidal estrogens.

In examining the potential sources of estrogenic activity in the culture media, we noted that phenol red, the commonly used pH indicator in tissue culture media, bears some structural resemblance to certain nonsteroidal estrogens (Fig. 1). As reported here, we find that phenol red is an estrogen and that, at the concentrations found in tissue culture media, it causes significant stimulation of cell proliferation and specific protein synthesis in estrogen-responsive cells. In addition, the antiestrogen suppression of cell proliferation under "control" conditions can be accounted for by the suppression of the phenol red-stimulated activity.

MATERIALS AND METHODS

Chemicals. The samples of phenol red used in the cell culture experiments were obtained from GIBCO (lot nos. 11P5152 and 65N1053) and Fisher (lot no. 5-983-8). Reversed-phase HPLC analysis of these samples (C_{18} column with 0.1% aqueous trifluoroacetic acid and acetonitrile) showed a major peak accounting for 75–95% of the material and having UV-visible spectra in acid and base consistent with phenol red.

The antiestrogens *trans*-tamoxifen and *trans*-4-hydroxytamoxifen were provided by Stuart Pharmaceuticals (Wilmington, DE). [2,4,6,7-^{3}H]Estradiol (100 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. The synthetic progestin [6,7-^{3}H]R 5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†Present address: Laboratoire Cancerologie Experimental, Faculte Medecine Secteur Nord, Boulevard Pierre Dramard, Marseille, France.
§To whom reprint requests should be addressed.

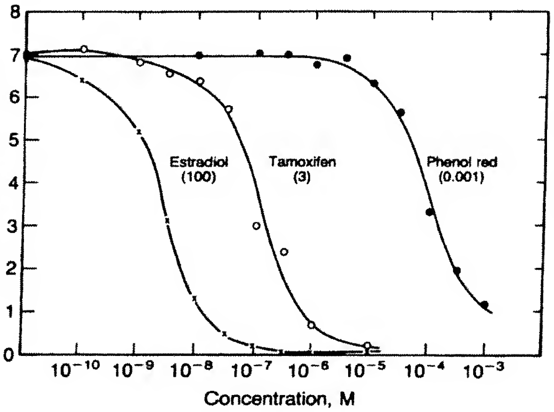


FIG. 2. Competitive binding assay of phenol red and tamoxifen. MCF-7 cytosol was incubated for 17 hr at 0–4°C with the indicated concentrations of competitor and 2.5 nM [^{3}H]estradiol. After incubation, bound radioactivity was determined by using hydroxylapatite. Numbers in parentheses indicate the relative binding affinity of each compound for receptor, with estradiol being set at 100.

dione; (87 Ci/mmol) was obtained from New England Nuclear. All media (phenol red-free and regular media containing phenol red), sera, and antibiotics used in cell cultures were obtained from GIBCO.

Cell Culture. MCF-7 cells, obtained from the Michigan Cancer Foundation (Detroit, MI), were grown in plastic T-150 flasks in Eagle's minimal essential medium (MEM) containing 5% dextran-coated charcoal-treated calf serum and other additives as described (6, 15). MDA-MB-231 cells, provided by the E.G. & G. Mason Research Institute (Worcester, MA), were grown in Leibovitz's medium L-15 supplemented as for MCF-7 cells plus glutathione (16 mg/l) and 5% calf serum (11). They were grown in the presence of 5% dextran-coated charcoal-treated calf serum for 2 weeks before use in cell proliferation experiments.

Cell Proliferation Experiments. To determine the effect of phenol red on cell proliferation, MCF-7 cells grown for 1 week before experiments in phenol red-free MEM supplemented as described above were harvested and seeded into T-25 flasks (*ca.* 1.5 × 10^5 cells per flask). The following day, cells from three flasks were harvested and counted with a Coulter Counter. Then the medium was changed to phenol red- and insulin-free MEM, which contained various concentrations of dextran-coated charcoal-treated calf serum, phenol red, tamoxifen, hydroxytamoxifen, estradiol, or ethanol vehicle (0.1%), and cell number was monitored as a function of time.

Preparation of Cytosol and Nuclear Extracts and Receptor Assays. Receptor preparations were made as described (6, 15), and the hydroxylapatite assay was used to determine the estrogen and progesterone receptor content of the cell extracts (6).

RESULTS

Binding Affinity of Phenol Red for the MCF-7 Estrogen Receptor. The binding affinity of phenol red, estradiol, and tamoxifen for the cytosol (180,000 × *g* for 30 min) MCF-7 estrogen receptor was determined by competitive binding analysis. Comparison of the concentrations needed to produce a 50% decrease in the specific binding of tritiated estradiol (Fig. 2) indicates that phenol red has an affinity 0.001% that of estradiol. Since under these conditions, estradiol has an equilibrium dissociation constant (K_d) of 2 × 10^{-10} M, the relative affinity of phenol red suggests a K_d of approximately 2 × 10^{-5} M. Although this affinity is low, the concentration of phenol red in the tissue culture medium used in culturing MCF-7 cells is very high, 30 μM.

Effect of Phenol Red on MCF-7 Cell Proliferation. To determine the effect of phenol red on cell proliferation, we measured the rate of proliferation of MCF-7 cells in complete medium ("regular MEM"), in medium lacking phenol red (phenol red-free MEM), and in phenol red-free MEM to which 30 μM phenol red was added. For each of these media, proliferation rate was determined at four different concentrations of steroid-stripped (treated with dextran-coated charcoal) calf serum, since serum is known to alter the rate of MCF-7 cell proliferation (8, 16–18) (Fig. 3).

In complete medium (regular MEM), which contains 30 μM phenol red, cell proliferation rate was highest at low serum concentration and was substantially reduced at the highest concentration of serum (20%). When phenol red was omitted from the medium (phenol red-free MEM), the rate of

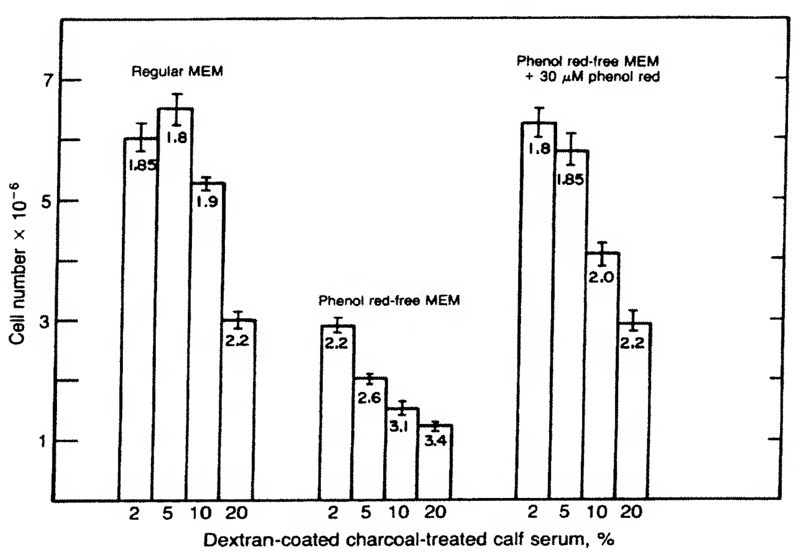


FIG. 3. Effect of phenol red on the proliferation of MCF-7 cells in the presence of different serum concentrations. Cells were grown in T-25 flasks in the presence of regular MEM, or phenol red-free MEM, or phenol red-free MEM containing 30 μM phenol red. Each medium was supplemented with the indicated concentration of dextran-coated charcoal-treated calf serum. Media were changed every other day; on day 8, triplicate flasks of cells were counted. Values represent the mean and range of the three cell numbers for each group. The numbers inside each bar indicate the cell-doubling time in days.

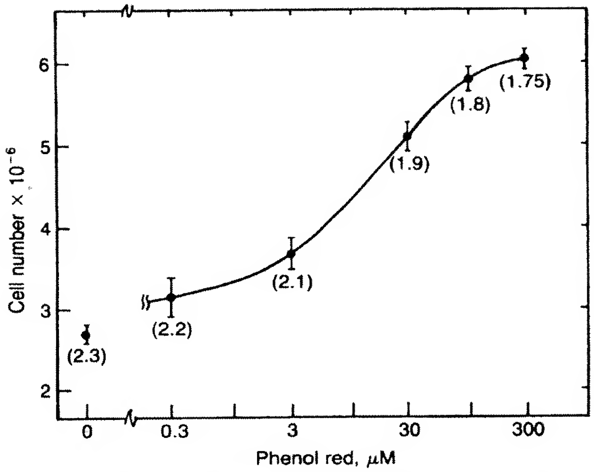


FIG. 4. Dose–response effect of phenol red on the proliferation of MCF-7 cells. Cells were grown in T-25 flasks in phenol red-free MEM containing 5% dextran-coated charcoal-treated calf serum and the indicated concentrations of phenol red. Growth was monitored exactly as described in Fig. 3.

cell proliferation was significantly decreased at all serum concentrations, relative to the corresponding rate in regular MEM. As expected, addition of 30 μM phenol red to the phenol red-free MEM media resulted in a restoration of proliferation rate to that seen in cells grown in the regular MEM medium, confirming that phenol red was the sole agent responsible for the accelerated growth rate. A serum concentration of 5% was selected for the next experiments.

Fig. 4 shows the dose–response effect of phenol red on MCF-7 cell proliferation. Phenol red stimulated the cell proliferation in a dose-dependent manner: 3 μM slightly increased the cell number versus control, and 30 μM phenol red was able to increase the cell number to about 200% of control, which was slightly below the maximum stimulation observed with 100 and 300 μM.

Table 1. Progesterone receptor concentrations in control and estradiol-treated MCF-7 cells grown in the presence or absence of phenol red

	Progesterone receptor levels	
Treatment	Sites per cell	pmol/mg of DNA
Without phenol red		
Control 1	1,915	0.71
Control 2	2,048	0.70
1 nM estradiol	30,546	11.3
1 nM estradiol	27,934	10.2
With phenol red		
Control 1	5,362	2.2
Control 2	5,085	2.0
1 nM estradiol	31,258	11.9
1 nM estradiol	26,693	10.9

Progesterone receptor levels in MCF-7 cells were determined after 5 days of growth in the presence or absence of added 1 nM estradiol in phenol red-free MEM or phenol red-free MEM supplemented with 30 μM phenol red. Both media contained 5% dextran-coated charcoal-treated calf serum. Fresh medium and hormone were added daily during the 5-day period. The cells were then harvested, fractionated, and assayed for progesterone receptor by utilizing 10 nM [^{3}H]R 5020 in the absence and presence of a 100-fold excess of radioinert R 5020. Each value represents data obtained from duplicate T-75 flasks of cells and is representative of two different experiments.

Stimulation of the Cellular Progesterone Receptor Levels by Phenol Red. Since progesterone receptor is an estrogen-stimulated protein (15, 19), we examined the effect of phenol red on progesterone receptor levels in MCF-7 cells. Basal progesterone receptor level was 3 times higher in control cells grown in medium containing 30 μM phenol red than in control cells grown in phenol red-free MEM (Table 1). Cells treated with 1 nM estradiol exhibited the same high level of progesterone receptor, regardless of whether phenol red was absent or present. Because of the differing control levels of progesterone receptor, the magnitude of the estradiol-stimulated induction of progesterone receptors was about 550% of

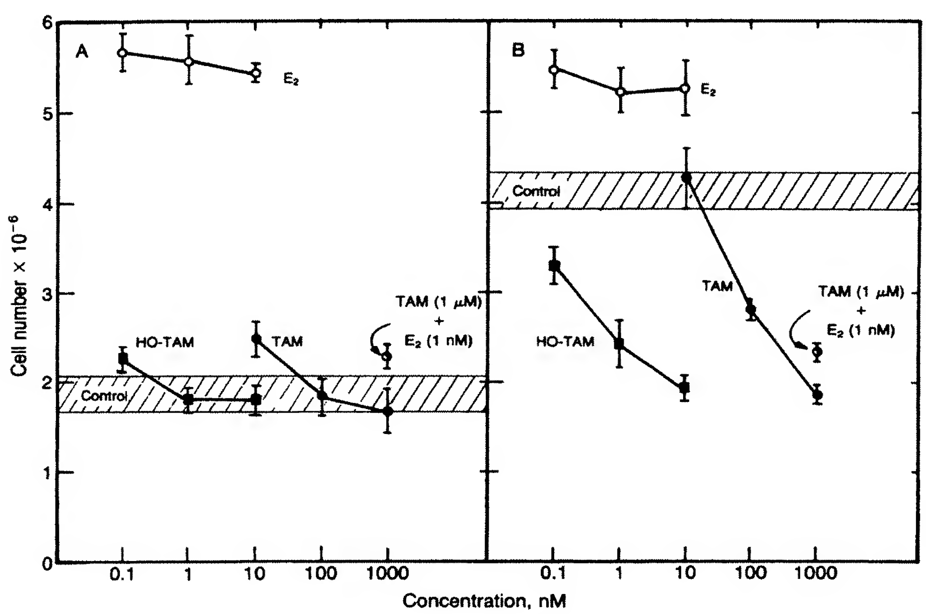


FIG. 5. Effect of estradiol and the antiestrogens tamoxifen and hydroxytamoxifen on the proliferation of MCF-7 cells in MEM tissue culture medium containing either no phenol red (*A*) or 30 μM phenol red (*B*). Cells were grown in T-25 flasks in phenol red-free MEM or in phenol red-free MEM to which 30 μM phenol red was added. Both media contained 5% dextran-coated charcoal-treated calf serum. Each medium was supplemented with the indicated concentration of estradiol (E_2) (○) or of tamoxifen (TAM) (●) or hydroxytamoxifen (HO-TAM) (■), or of 1 nM estradiol + 1 μM tamoxifen (TAM + E_2) (◐). Control cells received the 0.1% ethanol vehicle. Media and hormones were changed every other day, and, on day 8, triplicate flasks of cells were counted. Values represent the mean and range of the three cell numbers for each group. The horizontal hatched areas indicate the range of the control values.

control in the presence of phenol red and 1500% in the absence of phenol red.

Effect of Estrogen and Antiestrogens on MCF-7 Cell Proliferation in the Absence and Presence of Phenol Red. Since phenol red was shown to stimulate cell proliferation, we investigated the effect of estradiol and the antiestrogens tamoxifen and hydroxytamoxifen on cell proliferation in the absence or presence of 30 μM phenol red. As previously found (cf. Fig. 3), 30 μM phenol red increased the cell number to 200% of the phenol red-free control (Fig. 5, control levels). Estradiol (0.1–10 nM) increased the cell number to the same level with or without phenol red (Fig. 5, $+E_2$ data points). The percentage of estradiol-stimulation versus control was about 300% for cells grown in phenol red-free medium but only 130% in the presence of phenol red, because of the higher control level.

In the presence of phenol red (Fig. 5*B*), tamoxifen (10 nM to 1 μM) and hydroxytamoxifen (0.1–10 nM) decreased the cell number in a dose-dependent manner to 45% of the control. Also, 1 μM tamoxifen inhibited the cell proliferation stimulated by 1 nM estradiol, reducing the cell number to below that of the control. In contrast, when phenol red was omitted from the medium (Fig. 5*A*), neither of the antiestrogens inhibited cell growth to below that of the control. In fact, the lowest concentration of each antiestrogen appeared to cause a slight stimulation of cell proliferation. And, when tamoxifen (1 μM) was administered with estradiol (1 nM), tamoxifen inhibited estradiol-stimulated proliferation to the control level. In addition, it is of note that the level of suppression of control cell growth by antiestrogens when the cells were growing in the presence of phenol red is equivalent to the control growth rate obtained in the absence of phenol red. These results firmly suggest that antiestrogens only antagonize the phenol red- and E_2-stimulated cell proliferation, so that in the absence of estrogenic stimulation (phenol red-free, without estradiol), no growth suppression is observed with antiestrogens.

Effect of Phenol Red on Estrogen Receptor-Negative MDA-MB-231 Breast Cancer Cells. In order to confirm the estrogen receptor-mediated effect of phenol red, we examined the effect of 30 μM phenol red on MDA-MB-231 cells, which do not contain estrogen receptors and are reported to be estrogen- and antiestrogen-unresponsive (11, 20). Fig. 6 shows that these cells grew at the same rate in the presence and absence of phenol red (30 μM) and, likewise, that estradiol (1 nM) and tamoxifen (1 μM) did not have any effect on the growth of MDA-MB-231 cells.

DISCUSSION

We have found that phenol red possesses estrogenic properties: it binds to estrogen receptors with an affinity 0.001% that of estradiol; it stimulates cell proliferation in a dose-dependent manner, and it increases cellular progesterone receptor levels. Although phenol red is a low-affinity estrogen, it is present in tissue culture media at high concentrations. In the five media used most commonly for human breast cancer cells, its concentration is: 10 mg/liter (30 μM) for Eagle's MEM, Improved MEM, and Leibovitz L-15 media; 5 mg/liter (15 μM) for RPMI 1640 medium; or 15 mg/liter (45 μM) for Dulbecco's MEM. Thus, MCF-7 cells grown in phenol red-containing medium under apparently "control" conditions are, in fact, significantly estrogen-stimulated in terms of growth rate and progesterone receptor levels. The presence of phenol red might also influence the subcellular distribution of estrogen receptors in cells grown under conditions presumed to be free from estrogenic stimulation (21, 22).

Although the addition of estradiol to control cells grown in the presence of phenol red enhances cell proliferation and cellular progesterone receptor levels, a far greater "fold" stimulation is observed when estradiol is added to control cells grown in the absence of phenol red. Thus, the presence of phenol red masks part of the effect of estradiol and leads to underestimation of the response potential of the MCF-7 cells. This may explain the variable degree to which these cells are reported to be growth-stimulated by estradiol (5, 6, 20, 23, 24).

Although phenol red affects MCF-7 cell growth rate, it is clear that serum factors also modulate growth (8, 16–18). While the increased estrogen responsiveness of cells at high serum concentrations may be due in part to a decrease in the free phenol red concentration (increased protein binding), it appears that growth inhibitory factors are present in serum because, even in the absence of phenol red, MCF-7 cell growth rate decreases with increased serum concentrations (Fig. 3).

It has been a curious observation that significant levels of estrogen-induced protein and RNA species are observed in cells grown under "control" conditions and that in some cases these "basal" levels, assumed to be estrogen independent, are decreased by antiestrogen treatment (6, 23–27). Our finding that cells grown in the presence of phenol red are significantly estrogen-stimulated may account for the basal level of these estrogen-inducible species in "control" cells.

In this regard, it is well known that antiestrogens inhibit estrogen-stimulated cell proliferation and that this effect is estrogen receptor-mediated (4, 6, 11, 20, 28, 29). However, many published reports also show that antiestrogen treatment leads to a suppression of hormone-sensitive cell growth, even in the apparent absence of previous estrogen stimulation. Since this has been observed even in cases where the removal of estrogens from serum has been carefully documented (8, 19), some researchers have suggested that this suppressive action of antiestrogens might proceed by a mechanism not involving the estrogen receptor (12, 30).

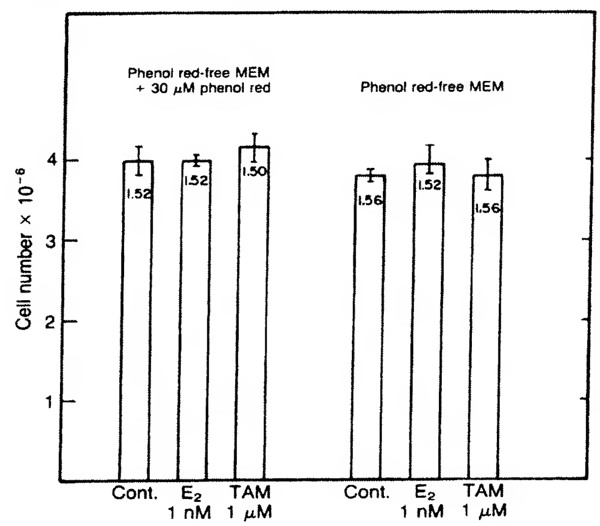


FIG. 6. Effect of phenol red on the proliferation of MDA-MB-231 human breast cancer cells. Cells were grown in T-25 flasks in phenol red-free MEM (*Right*) or phenol red-free MEM to which 30 μM phenol red was added (*Left*). Both media contained 5% dextran-coated charcoal-treated calf serum. Each medium was supplemented with 1 nM estradiol (E_2) or 1 μM tamoxifen (TAM) or 0.1% ethanol vehicle for the control (Cont.). Media were changed every other day, and, on day 8, triplicate flasks of cells were counted. Values represent the mean and range of the three cell numbers for each group. The numbers inside each bar indicate the cell-doubling time in days.

In our study, the results obtained in the presence of phenol red do not differ from these earlier findings: antiestrogens inhibited both estradiol-stimulated and control cell growth. However, in the absence of phenol red, antiestrogens inhibited estradiol-stimulated cell proliferation to the control level but did not decrease the growth of untreated, control cells. These results suggest that the growth-inhibitory effect of antiestrogens observed in the presence of phenol red is only due to the inhibition of the phenol red-stimulated cell proliferation. Our findings are in accord with studies performed in nude mice (31) in which antiestrogens inhibit estradiol-stimulated growth of tumors obtained by MCF-7 inoculation but do not alone lead to their regression.

The fact that MCF-7 cells grown in the presence of phenol red are already partially estrogen-stimulated, makes this system insensitive for assaying possible weak agonistic effects that may be demonstrated by antiestrogens under certain circumstances. Indeed, in the absence of phenol red, we do detect (Fig. 5) a very weak agonistic, growth-stimulatory effect of the antiestrogens tamoxifen and hydroxytamoxifen, but only at low concentrations. This *in vitro* observation appears to be consistent with some reports of weak stimulatory effects of tamoxifen on human tumor proliferation in nude mice (29, 31–33).

Our data clearly indicate that phenol red, ubiquitously used in tissue culture media, is significantly estrogenic and influences the evaluation of the effects of estrogens and antiestrogens on cell proliferation and protein synthesis. Its presence affects the basal level of hormone-stimulated responses and likewise the degree to which exogenous estrogen is able to stimulate these responses above the basal level. This activity of phenol red should clearly be considered in any studies utilizing estrogen-responsive cells.

We are grateful to Dr. R. D. Bindal for the HPLC analyses of phenol red samples. Support of this research from the National Institutes of Health (HHS 5RO1 CA 18119 to B.S.K. and HHS 5RO1 AM15556 to J.A.K.) is gratefully acknowledged. Y.B. was supported, in part, by a fellowship from Institut National de la Santé et de la Recherche Médicale.

1. Sato, G. H. & Ross, R. (1979) *Hormones and Cell Culture*, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Books A and B.
2. Sirbasku, D. A. & Leland, F. E. (1982) in *Biochemical Actions of Hormones*, ed. Litwack, G. (Academic, New York), Vol. 9, pp. 115–140.
3. Katzenellenbogen, B. S., Bhakoo, H. S., Ferguson, E. R., Lan, N. C., Tatee, T., Tsai, T. L. & Katzenellenbogen, J. A. (1979) *Recent Prog. Horm. Res.* **35**, 259–300.
4. Katzenellenbogen, B. S., Miller, M. A., Mullick, A. & Sheen, Y. Y. (1985) *Breast Cancer Res. Treat.* **5**, 231–245.
5. Aitken, S. C. & Lippman, M. E. (1985) *Cancer Res.* **45**, 1611–1620.
6. Katzenellenbogen, B. S., Norman, M. J., Eckert, R. L., Peltz, S. W. & Mangel, W. F. (1984) *Cancer Res.* **44**, 112–119.
7. Vignon, R., Terqui, M., Westley, B., Derocq, D. & Rochefort, H. (1980) *Endocrinology* **106**, 1079–1086.
8. Darbre, P., Yates, J., Curtis, S. & King, R. J. B. (1983) *Cancer Res.* **43**, 349–354.
9. Barnes, D. & Sato, G. (1980) *Cell* **22**, 649–655.
10. Edery, M., Imagawa, W., Larson, L. & Nandi, S. (1985) *Endocrinology* **116**, 105–112.
11. Miller, M. A. & Katzenellenbogen, B. S. (1983) *Cancer Res.* **43**, 3094–3101.
12. Sutherland, R. L., Foo, M. S., Greene, M. D., Waybourne, A. M. & Krozowski, Z. S. (1980) *Nature (London)* **288**, 273–275.
13. Sudo, K., Monsma, F. J., Jr., & Katzenellenbogen, B. S. (1983) *Endocrinology* **112**, 425–434.
14. Kon, O. L. (1983) *J. Biol. Chem.* **258**, 3173–3177.
15. Eckert, R. L. & Katzenellenbogen, B. S. (1982) *Cancer Res.* **42**, 139–144.
16. Page, M. J., Field, J. K., Everett, N. P. & Green, C. D. (1983) *Cancer Res.* **43**, 1244–1250.
17. Soto, A. & Sonnenschein, C. (1984) *Biochem. Biophys. Res. Commun.* **122**, 1097–1103.
18. Reiner, G. C. A., Nardulli, A., Norman, M. J., Mangel, W. F. & Katzenellenbogen, B. S. (1984) *Proc. Am. Assoc. Cancer Res.* **75**, abstr. 806, p. 204.
19. Horwitz, K. B. & McGuire, W. L. (1978) *J. Biol. Chem.* **253**, 2223–2229.
20. Lippman, M. E., Bolan, G. & Huff, K. (1976) *Cancer Res.* **36**, 4595–4601.
21. King, W. & Greene, G. L. (1984) *Nature (London)* **307**, 745–747.
22. Welshons, W. V., Lieberman, M. E. & Gorski, J. (1984) *Nature (London)* **307**, 747–749.
23. Butler, W. B., Kelsey, W. H. & Goran, N. (1981) *Cancer Res.* **41**, 82–88.
24. Butler, W. B., Kirkland, W. L., Gargala, T. L., Goran, N., Kelsey, W. H. & Berlinski, P. J. (1983) *Cancer Res.* **43**, 1637–1641.
25. Westley, B. & Rochefort, H. (1980) *Cell* **20**, 353–362.
26. Westley, B. H., May, F., Brown, T., Krust, A., Chambon, P., Lippman, M. & Rochefort, H. (1984) *J. Biol. Chem.* **259**, 10030–10036.
27. Brown, A. M. C., Jeltsch, J. M., Robert, M. & Chambon, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6344–6348.
28. Coezy, E., Borgna, J. L. & Rochefort, H. (1981) *Cancer Res.* **42**, 317–323.
29. Osborne, C. K., Boldt, D. H. & Estrada, P. (1984) *Cancer Res.* **44**, 1433–1439.
30. Faye, J. C., Jozan, S., Redeuilh, G., Baulieu, E. E. & Bayard, F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3158–3162.
31. Osborne, C. K., Hobbs, K. & Clark, G. M. (1985) *Cancer Res.* **45**, 584–590.
32. Satyaswaroop, P. G., Zaino, R. J. & Mortel, R. (1984) *Cancer Res.* **44**, 4006–4010.
33. Zaino, R. J., Satyaswaroop, P. G. & Mortel, R. (1985) *Cancer Res.* **45**, 539–541.

APPENDIX C

IN VITRO TOXICOLOGY ASSAY KIT
MTT BASED
Stock No. TOX-1
Store at 2-8 °C

Product Information

This kit is designed for determining cell number spectrophotometrically as a function of mitochondrial activity in living cells.

IT IS RECOMMENDED THAT THE ENTIRE PROTOCOL BE REVIEWED BEFORE STARTING THE ASSAY.

PRODUCT DESCRIPTION

Traditionally, the in vitro determination of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye. Alternative methods used are measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters and others which rely on dyes and cellular activity. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases.

The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT. Solutions of MTT, dissolved in medium or balanced salt solutions without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions. The crystals are dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

REAGENT
For Research Use Only.
Not for Use in Diagnostic Procedures.

KIT COMPONENTS

Catalog No.	Item	Quantity
M 5655	MTT 15 mg/vial in serum vial	5
M 8910	MTT Solubilization Solution 10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol, 125 ml	1

WARNING: Components in this kit should be carefully handled when using. **MTT** may be harmful if swallowed, inhaled or absorbed through skin. MTT may alter genetic material. **MTT SOLVENT** is flammable and corrosive.

PRODUCT STORAGE

Kit components should be stored at 2-8 °C.

PROCEDURE

The MTT method of monitoring in vitro cytotoxicity is well suited for use with multiwell plates. For best results, cells in the log phase of growth should be employed and final cell number should not exceed 10^6 cells/cm^2. Each test should include a blank containing complete medium without cells.

NOTE: Bacteria, mycoplasma and other microbial contaminants may also cleave the MTT tetrazolium ring. Cultures containing microorganisms should not be assayed using this method.

1. Remove cultures from incubator into laminar flow hood or other sterile work area.

2. Reconstitute each vial of MTT [M 5655] to be used with 3 ml of medium or balanced salt solution without phenol red and serum. Add reconstituted MTT in an amount equal to 10% of the culture medium volume.

3. Return cultures to incubator for 2-4 hours depending on cell type and maximum cell density. (An incubation period of 2 hours is generally adequate but may be lengthened for low cell densities or cells with lower metabolic activity.) Incubation times should be consistent when making comparisons.

4. After the incubation period, remove cultures from incubator and dissolve the resulting formazan crystals by adding an amount of MTT Solubilization Solution [M-8910] equal to the original culture medium volume.

5. Gentle mixing in a gyratory shaker will enhance dissolution. Occasionally, especially in dense cultures, pipetting up and down [trituration] may be required to completely dissolve the MTT formazan crystals.
6. Spectrophotometrically measure absorbance at a wavelength of 570 nm. Measure the background absorbance of multiwell plates at 690 nm and subtract from the 570 nm measurement.

   Tests performed in multiwell plates can be read using the appropriate type of plate reader or the contents of individual wells may be transferred to appropriate size cuvets for spectrophotometric measurement.

POSSIBLE SOURCES OF ERROR

1. Reconstituted MTT solution is stable for at least 6 months when stored frozen (-0 °C). Storage of reconstituted MTT solution at 2-8°C for more than 2 weeks may cause decomposition and yield erroneous results.

2. Microbial contamination will contribute to the cleavage of MTT and the formation of MTT formazan, yielding erroneous results.

3. Uneven evaporation of culture fluid in wells of multiwell plates may cause erroneous results.

4. High protein levels may form a precipitate when MTT Solubilization Solution is added. Samples with protein concentrations equivalent to 10% fetal bovine serum (4 mg protein/ml of sample) seem acceptable. Sera or serum products with higher protein concentrations may need to be used at lower percentages.

5. Media and salt solutions with phenol red can be used but will contribute to higher background absorbance and can decrease sensitivity.

REFERENCES

1. Mossman, T. [1983] Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65:55-63.
2. Denizot, F. and Lang, R. [1986] Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89:271.
3. Carmicheal, J. et al. [1987] Evaluation of a tetrazolium-based, semi-automated colorimetric assay: assessment of chemosensitivity testing. Cancer Research 47:936-942.4.
4. Edmondson, J. et al. [1988] A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. J. Tissue Cult. Methods 11:15-17.
5. Vistica, D. et al. [1991] Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. Cancer Research 51:2515-2520.
6. Takeuchi, H. et al. [1991] An application of tetrazolium (MTT) colorimetric assay for the screening of anti-herpes simplex virus compounds. J. Virol. Methods 33:61-71.

IN VITRO TOXICOLOGY ASSAY KIT
MTT BASED
Stock No. TOX-1
7H258